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ORIGINAL RESEARCH PAPER

Biotransformation of heterocyclic dinitriles by *Rhodococcus erythropolis* and fungal nitrilases

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Abstract 2,6-Pyridinedicarbonitrile (**1a**) and 2,4-pyridinedicarbonitrile (**2a**) were hydrated by *Rhodococcus erythropolis* A4 to 6-cyanopyridine-2-carboxamide (**1b**; 83% yield) and 2-cyanopyridine-4-carboxamide (**2b**; 97% yield), respectively, after 10 min. After 118 h, the intermediates **1b** or **2b** were transformed into 2,6-pyridinedicarboxamide (**1c**; 35% yield) and 2,6-pyridinedicarboxylic acid (**1d**; 60% yield) or 2-cyanopyridine-4-carboxylic acid (**2c**; 64% yield), respectively. The nitrilase from *Fusarium solani* afforded cyanocarboxylic acids **1e** and **2c** after 118 h (yields 95 and 62%, respectively). 3,4-Pyridinedicarbonitrile (**3a**) and 2,3-pyrazinedicarbonitrile (**4a**) were inferior substrates of nitrile hydratase and nitrilase.

Keywords Amidase · *Aspergillus niger* · *Fusarium solani* · Heterocyclic dinitriles · Nitrilase · Nitrile hydratase · *Rhodococcus erythropolis*

Introduction

Previously, we reported on biocatalysts suitable for the mild hydrolysis of heterocyclic nitriles. *A nitrilase* from *Aspergillus niger* exhibited high activities for 3- and 4-cyanopyridine (Kaplan et al. 2006a). The latter compound was hydrolyzed at the highest rate of all substrates tested, probably due to an electron withdrawing effect of the heteroatom. Different immobilized formulations of the nitrilase from *Fusarium solani* were also highly active towards the above nitriles (Vejvoda et al. 2006a). In addition, a nitrile hydratase from *Rhodococcus erythropolis* A4 (formerly *R. equi* A4) was useful for the preparation of amides from heterocyclic nitriles like cyanopyridines (Přepechalová et al. 2001).

Considering the potential of these enzymes for the production of heterocyclic acids or amides, we became interested in their activity towards heterocyclic dinitriles. Transformation of aliphatic and aromatic dinitriles attracted much attention due to its utility for the preparation of useful compounds bearing diverse functional groups (for reviews see Sugai et al. 1997 and Martínková and Mylerová 2003). In this work, we examined the feasibility of using nitrilase and nitrile hydratase for the transformations of 2,6-, 2,4- and 3,4-pyridinedicarbonitrile and 2,3-pyrazinedicarbonitrile.

Materials and methods

Chemicals

The substrates (see Fig. 1) were 2,6-pyridinedicarbonitrile (**1a**), 2,4-pyridinedicarbonitrile (**2a**),

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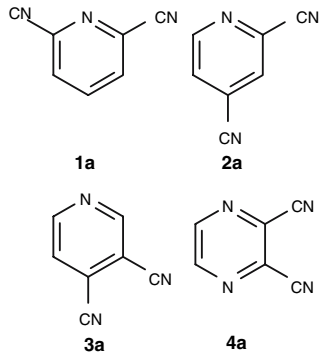

Fig. 1 Heterocyclic dinitriles used as substrates of nitrilases and nitrile hydratase/amidase

3,4-pyridinedicarbonitrile (**3a**), 2,3-pyrazinedicarbonitrile (**4a**) and benzonitrile. The authentic standards (see Fig. 2) were 2,6-pyridinedicarboxamide (**1c**) and 2,6-pyridinedicarboxylic acid (**1d**). All substrates and standards were purchased from Aldrich.

HPLC

Nitriles, amides and acids were analyzed by HPLC with a Novapak C_{18} column (4 μm, 150 × 3.9 mm; Waters). Benzonitrile, benzamide and benzoic acid were separated using a mobile phase composed of acetonitrile:water:phosporic acid (30:69.9:0.1) at a flow rate of 0.9 mol min^{-1} and 35°C. For separation conditions, retention times and spectral maxima of compounds **1a**–**1e**, **2a**–**2c**, **3a** and **4a** see Table 1.

Preparative HPLC was performed with a Nucleosil 120–5 C_{18} column (5 μm, 250 × 8 mm; Macherey-Nagel) at of 0.5 ml min^{-1} and ambient temperature. The mobile phase was composed of acetonitrile:water:acetic acid (10:89:1, by vol.).

16S rDNA sequencing

A fragment of 16S rDNA (881 bp) was amplified by PCR using the total genome DNA of the *Rhodococcus* strain A4 as a template and oligonucleotides 16SREF and 16SRER (Čejková et al. 2005) as primers. The sequence of the fragment was determined with ABI Prism 2100 sequencer (Perkin-Elmer). The sequence was found identical (100%) with the respective part of the homologous sequence of *R. erythropolis* 16S rDNA stored in the GenBank while there was only 96% identity with *R. rhodochrous* 16S rDNA and 95% identity with *R. equi* 16S rDNA. The strain A4, originally classified as *R. equi* according to biochemical tests, was thus reclassified as *R. erythropolis* on the basis of the similarity searches of 16S rDNA sequences.

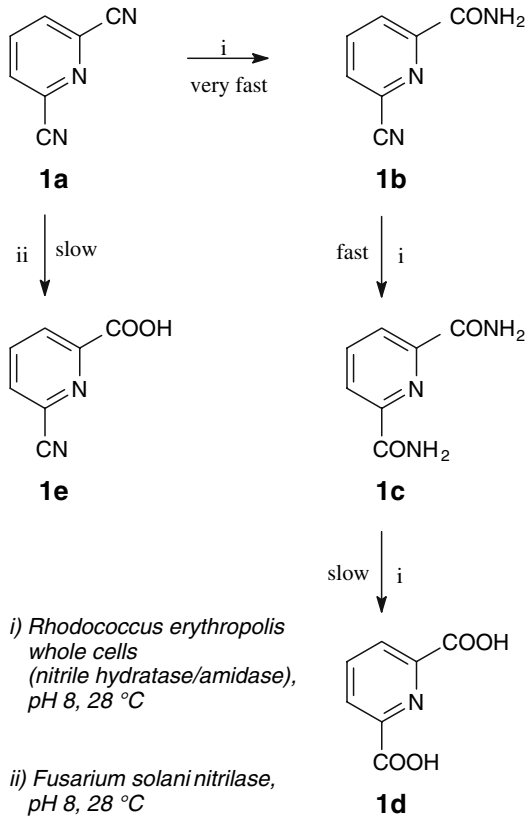

Fig. 2 Products formed from 2,6-pyridinedicarbonitrile (**1a**) by whole cells of *Rhodococcus erythropolis* (nitrile hydratase/amidase; i) and cell-free extract from *Fusarium solani* (nitrilase; ii)

Biocatalysts

Whole cells of *Rhodococcus erythropolis* A4 (deposited in the Culture Collection of Microorganisms, Masaryk University Brno) were grown as described by Martínková et al. (1998). The nitrile hydratase was partially purified as described by Přepechalová et al. (2001). The nitrilases from *Aspergillus niger* CCF 3411 (Culture Collection of Fungi, Charles University Prague, Czech Republic) and *Fusarium solani* CCF 3635 were used as a semi-purified enzyme (Kaplan et al. 2006a) and a cell-free extract (Vejvoda

Table 1 Analytical HPLC[a] of heterocyclic dinitriles and products of their enzymatic conversion

Compound	Retention time [min]	Local spectral maximum [nm]
2,6-Pyridinedicarbonitrile (**1a**)	10.5	221.6–273.5
6-Cyanopyridine-2-carboxamide (**1b**)	4.9	221.6–272.3
2,6-Pyridinedicarboxamide (**1c**)	3.1	272.3
2,6-Pyridinedicarboxylic acid (**1d**)	3.3	271.1
6-Cyanopyridine-2-carboxylic acid (**1e**)	3.9	221.6–271.1
2,4-Pyridinedicarbonitrile (**2a**)	6.9	281.8
2-Cyanopyridine-4-carboxamide (**2b**)	3.5	214.6–275.8
2-Cyanopyridine-4-carboxylic acid (**2c**)	5.7	279.4
3,4-Pyridinedicarbonitrile (**3a**)	6.6	222.8–284.1
2,3-Pyrazinedicarbonitrile (**4a**)	7.9	229.9–272.3

[a] The compounds were separated by using a Novapak C_{18} column (4 μm, 150 × 3.9 mm; Waters) and a mobile phase consisting of acetonitrile:water:phosphoric acid (8:91.9:0.1 by vol.) at 0.9 ml min^{-1} and 35°C

et al. 2006a), respectively. The enzyme assays are described below. The general procedure for preparative-scale biotransformations is described in Table 3.

Enzyme assays

Nitrile hydratase and nitrilase activities were assayed in 2 ml reaction mixtures composed 20 μmol benzonitrile in 50 mM Na_2HPO_4/KH_2PO_4 buffer, pH 8, and 5% (v/v) methanol as co-solvent. The reactions were started by the addition of an appropriate amount of the enzyme (approx. 0.2 U as assayed for benzonitrile). The nitrile hydratase- and nitrilase-catalyzed reactions proceeded at 32°C and 45°C, respectively, with shaking (850 rpm). After 10 min, the reactions were stopped by the addition of 20% (v/v) of 1 M HCl and the precipitated protein was removed by centrifugation. Benzamide and benzoic acid were determined by HPLC. Activities of the enzymes towards substrates **1a**–**4a** were assayed in the same way and substrate consumption was determined by HPLC. The product formation and substrate consumption was linear within the reaction time.

Product identification

Using authentic standards, products **1c** and **1d** were identified by HPLC and by UV spectra recorded by the PDA detector. Other products (**1b**, **1e**, **2b** and **2c**) were identified by NMR spectra recorded on a 400 MHz spectrometer (Varian unity Inova-400) in

Table 2 Substrate specificity of nitrilases from *Aspergillus niger* and *Fusarium solani*

Substrate	Relative activity (%)		
	Nitrile hydratase	Nitrilase from *F. solani*	Nitrilase from *A. niger*
Benzonitrile	100[a]	100[b]	100 [c]
2,6-Pyridinedicarbonitrile	48 ± 1	5 ± 0.9	30 ± 0.8
2,4-Pyridinedicarbonitrile	154 ± 7	7 ± 0.3	12 ± 0.2
3,4-Pyridinedicarbonitrile	23 ± 4	0	0.5 ± 0.1
2,3-Pyrazinedicarbonitrile[d]	22 ± 3	0	1 ± 0.7

[a] The specific activity of semi-purified enzyme for benzonitrile (32.5 U mg^{-1} protein) was taken as 100%

[b] The specific activity of the cell-free extract (9.8 U mg^{-1}protein) for benzonitrile was taken as 100%

[c] The specific activity of the semi-purified enzyme for benzonitrile (66 U mg^{-1} protein) was taken as 100%

[d] A minor part of the substrate decomposed spontaneously under the conditions used

Table 3 Yields of products (see Figs. 2 and 3) obtained from 2,6-pyridinedicarbonitrile (**1a**) and 2,4-pyridinedicarbonitrile (**2a**) by nitrile hydratase/amidase from *Rhodococcus erythropolis* and nitrilase from *Fusarium solani*

Substrate	Enzyme	Product	Yield [%][a] after reaction time of		
			10 min	2 h	118 h
1a	Nitrile hydratase/amidase[b]	**1b**	83	12	1
		1c	16	85	35
		1d	0	0	60
	Nitrilase[c]	**1e**	12	73	95
2a	Nitrile hydratase/amidase[b]	**2b**	97	78	32
		2c	1	7	64
	Nitrilase[c]	**2c**	1	12	62

[a] determined by HPLC (see Materials and methods and Table 1)

[b] The reaction mixtures (total volume 50 ml) consisted of 0.5 mmol substrate in 50 mM Na_2HPO_4/KH_2PO_4 buffer, pH 8, with 5% (v/v) methanol as co-solvent and whole cells of *R. erythropolis* (approx. 100 U nitrile hydratase). Reactions proceeded in 100 ml Erlenmeyer flasks at shaking (200 rpm) and 28°C. At intervals samples (0.2 ml) were withdrawn, acidified with 0.04 ml 1 M HCl, centrifuged and analyzed by HPLC (see Table 1). The reaction mixtures were centrifuged and products extracted from the supernatants with ethyl acetate (at pH 8.5–9 or 2–2.5 for amides or carboxylic acids, respectively). Alternatively, the supernatants were lyophilized overnight and the residues extracted with dry methanol. The products were purified by preparative HPLC (see Materials and methods)

[c] The reactions proceeded as described for nitrile hydratase/amidase but the enzyme was a cell-free preparation from *F. solani* (approx. 50 U nitrilase)

DMSO-d_6 at 30°C. The assignment was based on COSY, HMQC and HMBC experiments performed using the manufacturer's software. Residual solvent signals (DMSO: δ_H 2.50 ppm, δ_C 39.60 ppm) were used as internal standards.

Results and discussion

Substrate specificity of nitrile-converting enzymes towards heterocyclic dinitriles

Pyridine derivatives with cyano groups in meta-position (**1a**, **2a**) were good substrates of the nitrile hydratase from *R. erythropolis* (see Table 2). Steric hindrance by heteroatom probably decreased the reactivity of compound **1a**, which was transformed at approx. 3 times lower rate than compound **2a**. Not suprisingly, the activities of the nitrile hydratase towards dinitriles with cyano groups in *ortho*-position (**3a**, **4a**) were even lower. This enzyme was previously shown to be influenced by steric effects of *ortho*-substituents (Přepechalová et al. 2001).

The nitrilase from *F. solani* is more susceptible towards steric hindrances than that from *A. niger* (Kaplan et al. 2006b) and this observation is in accordance with lower activities of the former enzyme towards compounds **1a** and **2a** (see Table 2). This nitrilase showed no activity towards *ortho*-substituted compounds **3a** and **4a**, while the nitrilase from *A. niger* was able to hydrolyze them at a very low rate.

Products of enzymatic hydrolysis of heterocyclic dinitriles

The above experiments suggested the feasibility of preparative biotransformations of dinitriles **1a** and **2a** by both nitrile hydratase and nitrilase. Previously, we observed that both enzymes hydrolyzed a single cyano group in dicyanobenzenes (Přepechalová et al. 2001, Kaplan et al. 2006a, b). Therefore, we studied the mode of action of these enzymes towards pyridinedicarbonitriles. Whole cells of *R. erythropolis* were used as the source of nitrile hydratase in preparative biotransformations. This strain also exhibited amidase activity. A cell-free extract from *F. solani* was used for the preparative nitrilase-catalyzed biotransformations despite its lower specific activity for **1a** and **2a**, as this enzyme was available in large quantities from hyperinduced cultures (Vejvoda et al. 2006a). In addition, the nitrilase from *A. niger* often produced

large amounts of by-products-amides (Kaplan et al. 2006a) and, therefore, seemed to be less suitable for preparative biotransformation than the enzyme from *F. solani*, which gave nearly pure carboxylic acids (Vejvoda et al. 2006a).

The biotransformations of **1a** and **2a** were monitored by HPLC within 0–5 days. Compound **1a** was transformed into three major products by whole cells of *R. erythropolis*, while compound **2a** gave only two major products on incubation with this biocatalyst. The nitrilase from *F. solani* gave one major product from each substrate. The products were isolated and identified during the reaction (see Table 3). In general, purification by preparative HPLC was necessary due to cross-contamination of the products and unreacted nitriles. The isolated yields were not optimized in this procedure, the aim of which was to obtain pure biotransformation products for structure elucidation. Commercial authentic standards were available only for diamide **1c** and diacid **1d**. The reaction patterns for substrates **1a** and **2a** are shown in Figs. 2 and 3, respectively. Analytical yields of the products at different reaction times are given in Table 3.

The biotransformation of substrate **1a** catalyzed by nitrile hydratase gave cyano amide **1b** after 10 min. This intermediate was then hydrated into diamide **1c** within approx. 2 h. The following amidase-catalyzed reaction was slow. The end product of this reaction was dicarboxylic acid **1d**, which was the major product after 118 h. On the other hand, the reaction of **2a** showed a different course. The corresponding cyano amide **2b** was also rapidly formed at the initial stage (10 min) but afterwards slowly transformed into cyano carboxylic acid **2c** as the major product by the amidase (after 118 h).

The products of nitrilase-catalyzed reactions were identified as cyanocarboxylic acids **1e** and **2c**. The reactions of **2a** catalyzed by both nitrile hydratase and nitrilase were regioselective, only the nitrile group distant from the heteroatom being susceptible to hydration or hydrolysis.

Formation of amides as by-products by nitrilases

The production of amides by nitrilases is probably caused by an atypical cleavage of the reaction intermediate (Stevenson et al. 1992). It was

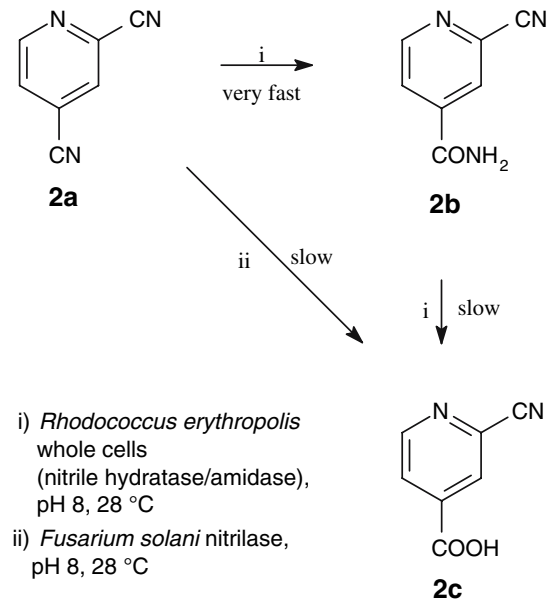

Fig. 3 Products formed from 2,4-pyridinedicarbonitrile (**2a**) by whole cells of *Rhodococcus erythropolis* (nitrile hydratase/amidase; i) and cell-free extract from *Fusarium solani* (nitrilase; ii)

especially pronounced for some substrates with electron-withdrawing groups (Effenberger and Osswald 2001, Kiziak et al. 2005). Nevertheless, this phenomenon is also strongly strain-dependent. The nitrilase from *F. solani* (Vejvoda et al. 2006a) formed no or only low amounts of amides from nitriles, as also observed in the present work. On the other hand, the nitrilase from *A. niger* (Kaplan et al. 2006a) formed high amounts of amides, especially from substrates with an electron-withdrawing heteroatom (2-cyanopyridine, 4-cyanopyridine). The substrates examined herein also contained electron withdrawing moieties (nitrogen heteroatom, cyano groups). Therefore, we examined the products formed by *A. niger* nitrilase from compounds **1a** and **2a**. Indeed, the corresponding amide was formed in both cases. The molar acid:amide ratio (**1c**:**1b**—9:1, **2c**:**2b**—7:3) was constant during the reaction. This side reaction may complicate the biocatalytical use of nitrilase from *A. niger*. On the other hand, the benefit of this enzyme is its higher specific activity towards sterically hindered substituted nitriles like **1a** and **2a**. Recently, we proposed to use a bienzymatic (nitrilase-amidase) reaction to remove the by-product amide (Vejvoda et al. 2006b).

Conclusion

The enzymatic hydration or hydrolysis of 2,6-pyridinedicarbonitrile and 2,4-pyridinedicarbonitrile provided access to heterocyclic compounds bearing multiple functional groups in their molecules. These compounds can serve as useful intermediates for chemical synthesis. The product type (cyano amide, cyano acid) could be controlled by the choice of an appropriate enzyme and by the reaction time.

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Appendix

6-Cyanopyridine-2-carboxamide (**1b**) ^{1}H NMR (399.87 MHz, DMSO-d_6, 30°C): 7.830 (1 H, br s, NH_2-u), 8.211 (1H, dd, J = 7.6, 2.2 Hz, H-5), 8.22 (1H, br s, NH_2-d), 8.238 (1 H, dd, J = 7.6, 7.0 Hz, H-4), 8.294 (1H, dd, J = 7.0, 2.2 Hz, H-3). ^{13}C NMR (100.55 MHz, DMSO-d_6, 30°C): 117.09 (6-CN), 125.88 (C-3), 131.31 (C-5), 131.46 (C-6), 139.75 (C-4), 151.91 (C-2), 164.49 (2-CO).

6-Cyanopyridine-2-carboxylic acid (**1e**) ^{1}H NMR (399.87 MHz, DMSO-d_6, 30°C): 8.231 (2 H, m, H-4, H-5), 8.296 (1 H, m, H-3). ^{13}C NMR (100.55 MHz, DMSO-d_6, 30°C): 117.01 (6-CN), 128.21 (C-3), 131.64 (C-5), 132.57 (C-6), 139.63 (C-4), 150.23 (C-2), 164.77 (2-CO).

2-Cyanopyridine-4-carboxamide (**2b**) ^{1}H NMR (399.87 MHz, DMSO-d_6, 30°C): 7.948 (1 H, br s, NH), 8.078 (1 H, dd, J = 5.1, 1.7 Hz, H-5), 8.347 (1 H, dd, J = 1.7, 0.9 Hz, H-3), 8.370 (1 H, br s, NH), 8.904 (1 H, dd, J = 5.1, 0.9 Hz, H-6). ^{13}C NMR (100.55 MHz, DMSO-d_6, 30°C): 117.29 (2-CN), 125.49 (C-5), 126.69 (C-3), 133.26 (C-2), 142.92 (C-4), 152.11 (C-6), 164.55 (4-CO).

2-Cyanopyridine-4-carboxylic acid (**2c**) ^{1}H NMR (399.87 MHz, DMSO-d_6, 30°C): 8.079 (1 H, dd, J = 4.9, 1.3 Hz, H-5), 8.296 (1 H, s, H-3), 8.872 (1 H, d, J = 4.9 Hz, H-6). ^{13}C NMR (100.55 MHz, DMSO-d_6, 30°C): 117.37 (2-CN), 126.85 (C-5), 128.05 (C-3), 133.25 (C-2), 143.11 (C-4), 152.07 (C-6), 164.82 (4-CO).

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